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13. ABSTRACT ***(Maximum 200 Words)***

We have successfully developed a chromatin immunoprecipitation (ChIP) assay system, utilizing an MCF-7 cell line to investigate the involvement of Uba3 in transcription within the promoter of endogenous ER target genes. We have successfully observed recruitment of ERα, SRC-1, and p300 to ER binding sites within the pS2 and c-Myc promoters. During the development phase of the assay system, we expressed a GST-UBA3 fusion protein in *E. coli*, and purified Uba3 for antibody production in rabbit. The antibody from serum was purified using full length Uba3 as an epitope to achieve pure antibody species, specifically recognizing Uba3. Western blot analysis indicates that the antibody recognizes a protein with molecular weight 66kDa in MCF-7 whole cell lysate, 16kDa higher than its predicted weight of 50kDa. Uba3 is known to be covalently attached to NEDD8, and Western blot analyses of NEDD8 and Uba3 suggests that the 66kDa protein is likely to be covalently coupled NEDD8-Uba3. We are in the process of using this antibody for ChIP assay analysis. Development of a Uba3-specific antibody is essential for future work detailed in the objectives section of the original proposal.

14. SUBJECT TERMS
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Introduction

Estrogen and progesterone regulate the growth and development of the mammary gland via a signaling cascade that is initiated through binding to their cognate nuclear hormone receptors (NHRs), the estrogen receptor (ER) and progesterone receptor (PR), members of the ligand-inducible transcription factor superfamily (1,2). ER and PR-targeted genes are further regulated by recruitment of coregulator proteins, which positively affect (coactivators) or negatively affect (corepressors) ligand-activated transcription (3).

We have identified human ubiquitin-activating enzyme 3 (hUba3) as a progesterone receptor (PR) interacting protein in a yeast 2-hybrid assay. Human Uba3 is a member of the NEDD8 (neural precursor cell-expressed developmentally down-regulated) ubiquitin-like protein modification pathway (4). The NEDD8 pathway functions through a pathway-specific E1 enzyme (a heterodimer of hUba3 and APP-BP1(amyloid precursor protein binding-protein 1)) and E2 enzyme (Ubc12 (ubiquitin-conjugating enzyme 12)), but lacks an E3 enzyme (4). We have previously shown that hUba3 as well as its heterodimeric binding partner, APP-BP1, were able to enhance ligand-activated ERα, PR-B, GR, AR, and TRβ and RARα-dependent transcription in HeLa cells. By a classic reverse-squelching experiments, we demonstrated that both hUba3 and APP-BP1 exist in limiting quantities within the cellular milieu. Interestingly, hUba3 and APP-BP1 do not appear to contain appreciable intrinsic activation functions nor demonstrate direct physical associations with PR-B, suggesting these proteins may differ from other previously described nuclear receptor cofactors. Mutation of the NEDD8 acceptor site within hUba3 reduces its ability to coactivate PR, demonstrating the importance of the enzyme function of hUba3 in its ability to function as a coactivator. Furthermore, dominant negative Ubc12, the ubiquitin-conjugating enzyme of the NEDD8 pathway, significantly reduces progesterone-activated transcription of a PR-responsive reporter gene in T-47D cells.

The Cullin proteins (Cul-1,-2,-3,-4a,-4b, and 5) have been the only identified target proteins of the NEDD8 modification pathway (5). The Cullin proteins are an integral component of the SCF ubiquitin-protein (E3) ligase (a complex of Skp1, Cullin-x, and an F-box protein). Modification of the Cullin proteins by NEDD8 appears to control the ubiquitin ligase activity of the SCF complex by recruiting a ubiquitin-conjugating (E2) enzyme used by this complex, named Cdc34 (6). Since the E2-E3 components are juxtaposed to one another, it is thought that proximity contributes to the enhancement of SCF complex function. Thus, the NEDD8 pathway may contribute to degradation of components involved in transcription via the SCF complex. Several lines of evidence support the possibility that degradation is necessary for transcription to proceed (7,8).

Since the characterization experiments proposed under "Task 1" demonstrate unique effects of hUBa3 on receptor activity, we decided to use chromatin immunoprecipitation (ChIP) assays to characterize the involvement of hUba3 in transcription from endogenous estrogen-regulated target genes, pS2 and c-Myc, in an MCF-7 cell line.

Body

We have previously reported the characterization of hUba3 and APP-BP1 as nuclear receptor cofactors capable of functioning as coactivators in transient transfection assays in HeLa cells. Based on experiments with hUba3 and Ubc12, we concluded that the NEDD8 pathway may play an important role in nuclear receptor-dependent transcription. Since we were not able identify any physical interactions between hUba3 and PR *in vitro* and *in vivo*, it was thought that hUba3 may interact with other protein components involved in transcription. We therefore developed a chromatin immunoprecipitation (ChIP) assay system to characterize the involvement of hUba3 in transcription from endogenous estrogen-regulated target genes, pS2 and c-Myc, in an

MCF-7 cell line. This experiment serves two important purposes not immediately apparent. First, if hUba3 interacts with proteins involved in transcription, the ChIP assay will enable us to conclude that it is recruited during transcription but interacts with proteins other than the nuclear receptor, ERα. Second, it will provide enormous detail of the kinetics occurring during transcription not able to be deciphered using a transient transfection system.

Figure 1a and 1b are graphical representations of the promoter sequences of the pS2 and c-Myc genes, respectively, that were studied when we designed our ChIP assay. The underlined areas indicate the ERα binding sites that are thought to be significant in eliciting an estrogenic response. Primers designed for the ChIP assay are shown as blue-colored nucleotides. Figure 1c demonstrates that ERα is recruited to the promoter region of pS2 in a ChIP assay experiment following administration of estradiol. ERα was also shown to be recruited to the c-Myc promoter in a similar manner (data not shown). The estrogen receptor is recruited to the promoter regions within 25 minutes, but the signal diminishes by 125 minutes. Other proteins known to be involved in transcription, such as SRC-1 and p300, were also demonstrated to be recruited in a similar manner to ER on the pS2 promoter, indicating that the diminished signal is not specific to ERα (Figure 1d). It has been demonstrated that ERα undergoes estrogen-dependent degradation by several groups, and this degradation appears to be an integral aspect of transcription. We observed that protein levels of ERα gradually diminish over the same time period reported in the ChIP assay, strongly suggesting that the diminished signal observed in the ChIP assay with ERα, as well as SRC-1 and p300, is likely due to the degradation of ERα (Figure 1E).

We next wanted to address the involvement of hUba3 in transcription using the ChIP assay, so we needed an antibody specifically recognizing hUba3 and capable of immunoprecipitating the desired protein. No commercial antibodies have been found for hUba3 to date, although Santa Cruz® has antibody for yeast Uba3. We strongly desired a hUba3 antibody for the ChIP assay, and also for future work analyzing expression of hUba3 in human breast cancer cell lines and breast tumors. During the optimization phase of the ChIP assay, we used the GSTUBA3 construct (previously described for *in vitro* pulldown assays with ^{35}S-Methionine labeled APP-BP1) to purify Uba3 to be used as an immunogen to generate an anti-hUba3 antibody in rabbit. When induced by IPTG in *E. coli*, GSTUba3 is expressed as a full length 80kDa protein with little or no degradation products, and is easily purified on glutathione sepharose beads (Figure 2a). Furthermore, GST-Uba3 is fully cleavable by thrombin and soluble following dialysis in a buffer suitable for injection in rabbit (Figure 2b). Unfortunately, the first attempt to raise an antibody in rabbit was unsuccessful because the rabbit died following the third immunization booster. We quickly prepared more immunogen and again attempted the same procedure in rabbit. Serum collected following the final immunization booster was used to purify the hUba3 antibody. Figure 2c shows that the antibody purification procedure yielded a pure Ab, with heavy and light chains clearly distinguishable on a coomassie blue stained gel. In Western blot analysis of MCF-7 and HeLa whole cell lysate, we noticed that the antibody recognizes a ~66kDa protein, which is not the predicted molecular weight of hUba3 (Figure 2D and data not shown). The apparent differences in expected and observed molecular weights may be due to post-translational modifications, or more likely, hUba3 covalently-coupled to NEDD8. Western blot analysis of both hUba3 and NEDD8 suggests that the ~66kDa is covalently coupled Uba3-NEDD8 (Figure 2D).

We have not been able to find a buffer suitable for immunoprecipitation as of yet. Commonly used RIPA buffer, containing 0.1% SDS, does not appear to immunoprecipitate hUba3 (data not shown). We are currently adjusting the concentration of detergents used in immunoprecipitation buffer to achieve a specific antibody-antigen interaction. We therefore are not able to conclude if hUba3 is directly involved in transcription using the ChIP assay. A few

additional months are needed to optimize steps in the ChIP assay protocol dealing with the hUba3 antibody.

Generating an antibody against hUba3 will also be useful for other experiments outlined in the original proposal. For instance, we proposed to examine the expression of hUba3 and affected cell proliferation proteins in breast carcinoma cell lines. As mentioned above, the NEDD8 pathway appears to be important in regulating the SCF complex, an E3 ligase complex shown to be responsible for degrading important proteins implicated in breast cancer such as $p27^{KIP1}$ and IκBα (9,10,11). We will utilize the hUba3 antibody to analyze the correlation between Uba3 and these candidate proteins. Additionally, we proposed to examine the expression of hUba3 in human breast tumor biopsy samples, while analyzing several clinocopathological parameters, including tumor grade and ER/PR status. Again, the hUba3 antibody we have generated will be important in these studies. To support the results observed with the antibody, other methods of analysis will be used such as Ribonuclease protection assay (RPA) and *in situ* hybridization.

Key Research Accomplishments

- We have successfully developed a chromatin immunoprecipitation (ChIP) assay to study the recruitment and dynamics of proteins involved in estrogen-dependent transcription on endogenous pS2 and c-Myc promoters in MCF-7 cells.

- We have successfully generated and purified an antibody capable of specifically recognizing hUba3 in Western blot analyses. We are currently optimizing immunoprecipitation buffers to be used with the hUba3 antibody for other experiments.

- (in progress) Determine if hUba3 is involved in transcription using the ChIP assay model system and hUba3 antibody mentioned above.

Reportable Outcomes

None

Conclusions

We have successfully developed a chromatin immunoprecipitation (ChIP) assay to study the recruitment and dynamics of proteins involved in estrogen-dependent transcription on endogenous pS2 and c-Myc promoters in MCF-7 cells. We have shown ERα, SRC-1, and p300 recruitment to the pS2 promoter within 25 minutes following administration of estradiol, but the observed signal became diminished by 125 minutes. This observed diminished recruitment is likely due to estrogen-mediated degradation of the estrogen receptor by the 26S proteasome.

We have successfully generated and purified an antibody capable of specifically recognizing hUba3 in Western blot analyses. We were able to show that in MCF-7 and HeLa cells, hUba3 is expressed abundantly, and although the molecular weight of hUba3 is higher than predicted, we have shown that this higher molecular weight protein is likely NEDD8-coupled hUba3. This result suggests that the NEDD8 pathway is important to MCF-7 intracellular processes. In agreement this, we have previously shown the importance of the NEDD8 pathway in transcription using T-47D and HeLa cells.

The hUba3 antibody will be useful in the future for the other experiments outlined in the original proposal. These experiments include examining the expression of hUba3 and affected cell proliferation proteins in breast carcinoma cell lines. Additionally, we will examine the

expression of hUba3 in human breast tumor biopsy samples, while analyzing several clinocopathological parameters, including tumor grade and ER/PR status.

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Appendix

A.

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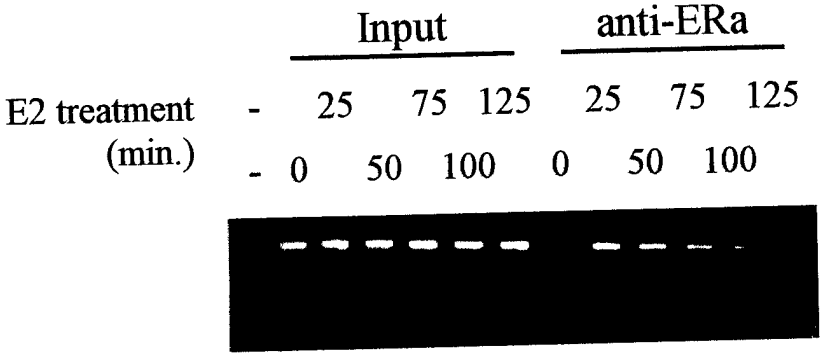

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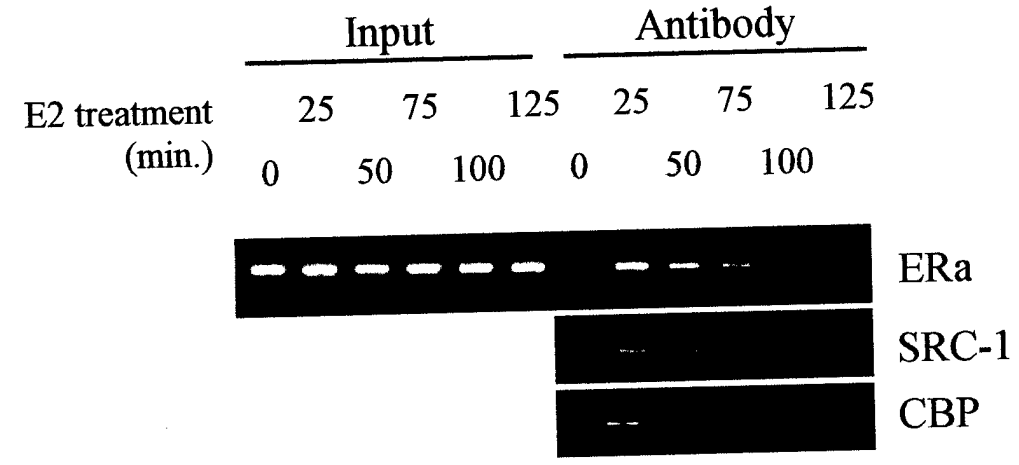

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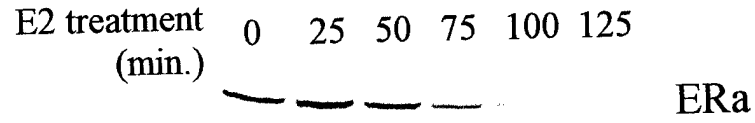

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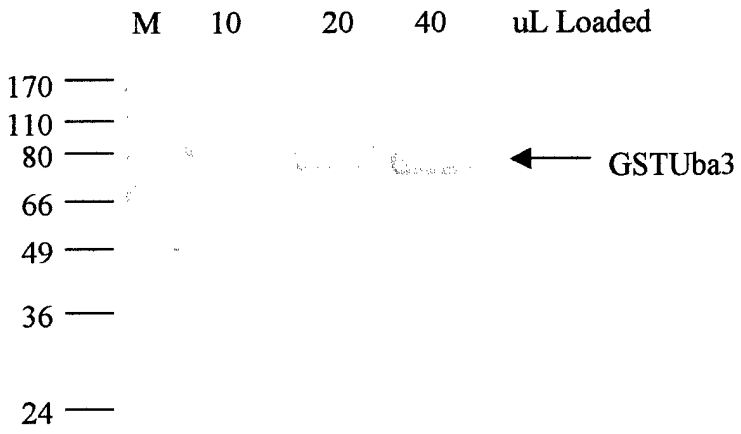
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GSTUba3

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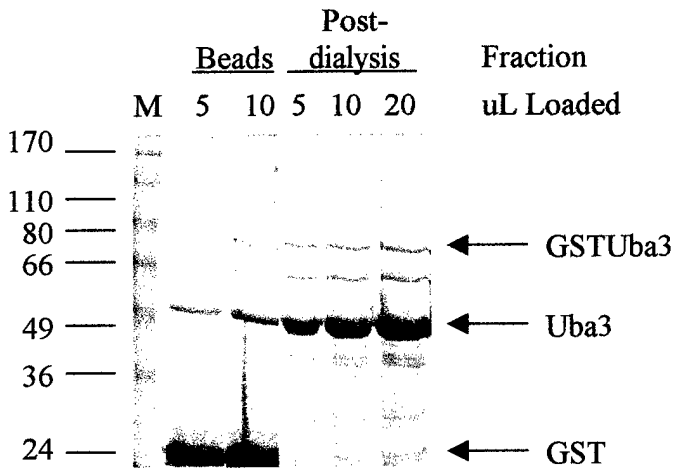
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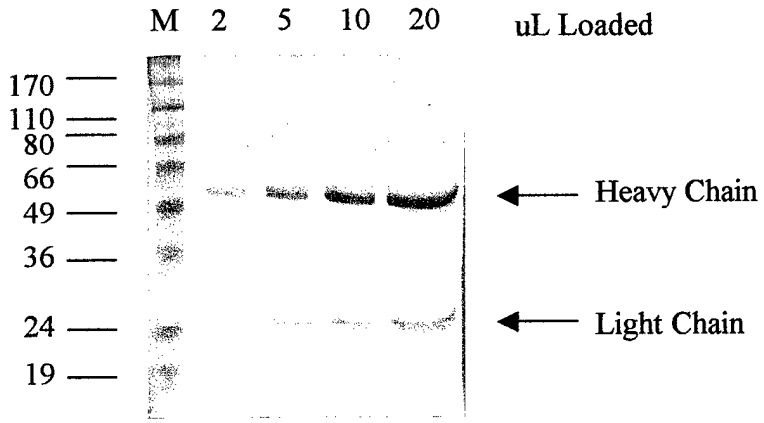
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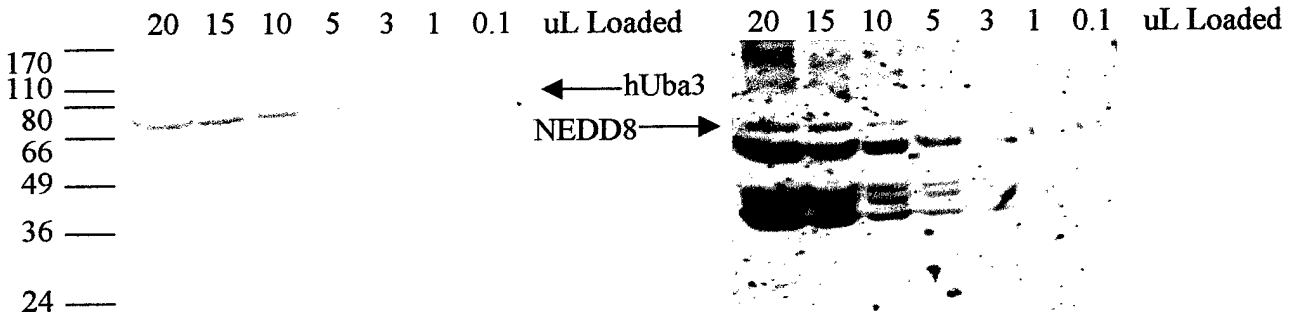
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24
hUba3
NEDD8

Figure 1. Dynamics of ERα, SRC-1, and p300 on the pS2 promoter in a chomatin immunoprecipitation (ChIP) assay. A, genomic organization of the pS2 promoter. Previously identified estrogen receptor binding sites are illustrated by underlined nucleotides. Transcription and translation start sites are illustrated by red-colored nucleotides. Primers used in the ChIP assay are illustrated by blue-colored nucleotides. B, genomic organization of the c-Myc promoter. Illustrations are the same as in the pS2 promoter. C, ChIP assay using the pS2 promoter primers and ERα antibody. The input lanes represent equal soluble chromatin used per time point. D, ChIP assay using the pS2 promoter primers and ERα, SRC-1, and p300 antibodies. The input lanes represent equal soluble chromatin used per time point. E, Western blot of MCF-7 whole cell lysate demonstrating that ERα is degraded following administration of estradiol.

Figure 2. Generation of a hUba3-specific antibody. A, GSTUba3 is expressed as a full-length 80kDa protein in *E. coli*. B, GSTUba3 was cleaved with 1U thrombin. The glutathione sepharose beads were extracted three times, and the eluates pooled. GST remains bound to the glutathione sepharose beads, while Uba3 is removed with the extractions. GSTUba3 remains soluble in the buffer used for immunization. C, rabbit serum was used to purify the hUba3 antibody. The antibody was eluted from the column and the eluates pooled. Heavy chain and light chain components of the hUba3 antibody are visualized. D, MCF-7 whole cell lysate was loaded on a SDS polyacrylamidegel and transferred overnight for Western blot analysis with the hUBa3 antibody (Left). The same blot was stripped, and blotted with NEDD8 antibody (right).